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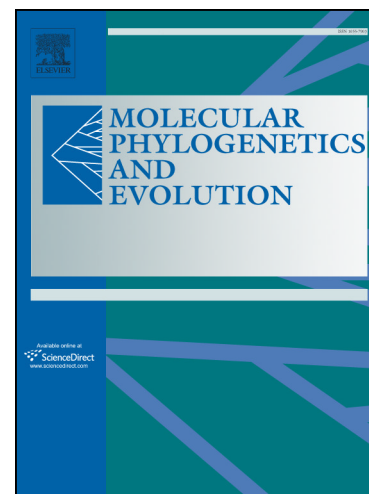
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Cryptic Lineage Differentiation Among Indo-Pacific Bottlenose Dolphins (*Tursiops aduncus*) in the Northwest Indian Ocean

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Graphical abstract

Highlights

- Discovery of a new lineage of *Tursiops aduncus* in the Indian Ocean.
- Divergence times within *Tursiops aduncus* coincide with Pleistocene glacial periods.
- Vicariance events in the northwest Indian Ocean and Australasia are proposed.

Abstract

Phylogeography can provide insight into the potential for speciation and identify geographic regions and evolutionary processes associated with species richness and evolutionary endemism. In the marine environment, highly mobile species sometimes show structured patterns of diversity, but the processes isolating populations and promoting differentiation are often unclear. The Delphinidae (oceanic dolphins) are a striking case in point and, in particular, bottlenose dolphins (*Tursiops* spp.).

Understanding the radiation of species in this genus is likely to provide broader inference about the processes that determine patterns of biogeography and speciation, because both fine-scale structure over a range of kilometers and relative panmixia

over an oceanic range are known for *Tursiops* populations. In our study, novel *Tursiops* spp. sequences from the northwest Indian Ocean (including mitogenomes and two nuDNA loci) are included in a worldwide *Tursiops* spp. phylogeographic analysis. We discover a new ‘*aduncus*’ type lineage in the Arabian Sea (off India, Pakistan and Oman) that diverged from the Australasian lineage ~261 Ka. Effective management of coastal dolphins in the region will need to consider this new lineage as an evolutionarily significant unit. We propose that the establishment of this lineage could have been in response to climate change during the Pleistocene and show data supporting hypotheses for multiple divergence events, including vicariance across the Indo-Pacific barrier and in the northwest Indian Ocean. These data provide valuable transferable inference on the potential mechanisms for population and species differentiation across this geographic range.

Keywords: Phylogeography, Pleistocene, Taxonomy, Conservation, *Tursiops aduncus*, Indian Ocean

1. Introduction

During the Pleistocene, rapid and dramatic climatic fluctuations generated extensive environmental change that would have influenced the temporal and spatial distribution of taxa over glacial cycles (Hofreiter and Stewart, 2009; Stewart *et al.*, 2010). In the marine environment, fluctuations in sea level changed coastal topography and caused patterns of isolation between areas of available habitat (e.g. Gaither and Rocha, 2013). Oscillations in climate also affected oceanographic processes, such as the reduction and intensification of monsoon systems associated with upwelling (Wang *et al.*, 1999a), which could have contributed to the spatio-

genetic structure and taxonomic variation in marine species. In the coastal waters of the northwest Indian Ocean there is high productivity off the Arabian Peninsula (Singh *et al.*, 2011; Banse and McClain, 1986; Bauer *et al.*, 1991; Burkill, 1999; Kindle and Arnone, 2001) and freshwater influx from rivers (e.g. the Indus delta), carrying large amounts of organic material (Longhurst, 2006). This, unique, heterogeneous environment has the potential to promote habitat dependencies or resource specialisations (e.g. Hoelzel, 1998b).

In this study we focus on the radiation of diversity in the genus *Tursiops*, in the sub-family Delphininae. Species within this group radiated recently, making genetic resolution difficult due to incomplete lineage sorting (retention of ancestral polymorphisms) and other confounding factors (e.g. Amaral *et al.*, 2012a). Species within this group have high dispersal ability yet often exhibit genetic structure over unexpectedly small spatial scales (e.g. Natoli *et al.*, 2004; Natoli *et al.*, 2008; Andrews *et al.*, 2010; Fernández *et al.*, 2011). Various studies have shown that genetic sub-division within these delphinid species is often associated with environmental heterogeneity (e.g. Bilgmann *et al.*, 2008; Natoli *et al.*, 2005; Natoli *et al.*, 2008; Andrews *et al.*, 2010; Mendez *et al.*, 2011) and/or historical climatic or geological events (e.g. Amaral *et al.*, 2012b; Moura *et al.*, 2013; Louis *et al.*, 2014; Moura *et al.*, 2014). As top predators, the pattern of genetic differentiation between populations of coastal delphinids may provide an insight into the broader ecological changes happening in the coastal waters of the Indian Ocean over time (see Fontaine *et al.*, 2007). Evolutionary endemism of marine mammal species has been documented in the region previously (e.g. Jefferson and Van Waerebeek, 2002; Mendez *et al.*, 2011; Minton *et al.*, 2011; Amaral *et al.*, 2012b; Mendez *et al.*, 2013; Pomilla *et al.*, 2014).

The taxonomy of bottlenose dolphins, *Tursiops* spp. has been the subject of much discussion (e.g. IWC, 2016). Although more work is needed (see Reeves *et al.*, 2004), resolution is improving, with the genus receiving much taxonomic attention in recent decades (e.g. Mead and Potter, 1990; Ross and Cockcroft, 1990; Hoelzel *et al.*, 1998; Wang *et al.*, 1999b; Möller and Beheregaray, 2001; Kemper, 2004; Natoli *et al.*, 2004; Charlton-Robb *et al.*, 2011; Moura *et al.*, 2013; IWC, 2016). The genus encompasses at least two species, the common bottlenose dolphin, *T. truncatus* and the Indo-Pacific bottlenose dolphin, *T. aduncus* (LeDuc *et al.*, 1999; Wang *et al.*, 1999b; 2000). There is recent support for a third species, the Burrunan dolphin, *T. australis*, from southern Australia (Charlton-Robb *et al.*, 2011) and further division within the *T. aduncus* group to include distinct lineages off South Africa, Australasia (Natoli *et al.*, 2004; Moura *et al.*, 2013) and possibly Bangladesh (Amaral *et al.*, 2016). Analysis of mtDNA from the *T. aduncus* holotype specimen (Red Sea) revealed it to be a match for the South African *T. aduncus* (Perrin *et al.*, 2007). Within the *T. truncatus* lineage, further division into regional ecotypes occupying coastal or pelagic habitat is recognised (Mead and Potter, 1995; Hoelzel *et al.*, 1998; Torres *et al.*, 2003). Regional patterns suggest that offshore *T. truncatus* can provide a source for colonizing coastal habitats (Tezanos-Pinto *et al.*, 2009; Richards *et al.*, 2013), though the broader pattern suggests a relatively recent radiation of the offshore populations (see Moura *et al.* 2013).

Patterns of divergence within bottlenose dolphins, and reconstructions of ancestral biogeography, suggest a coastal and Australasian origin for the *Tursiops* genus (Moura *et al.*, 2013). The South African *T. aduncus* (hereafter referred to as the holotype lineage) and the Australasian lineage diverged during the Pleistocene ~327 Ka (Moura *et al.*, 2013). To date, few phylogenetic studies have incorporated genetic

data from bottlenose dolphins in the northwest Indian Ocean. A study by Särnblad *et al.*, (2011; *in review*) showed that coastal bottlenose dolphins off Oman ($n = 4$) grouped with the holotype lineage of *T. aduncus*. Sightings data from the broader region suggest the presence of both coastal and pelagic *Tursiops* species; the latter recognized as *T. truncatus* based on morphology (Ponnampalam, 2009; Minton *et al.*, 2010) and mtDNA markers ($n = 13$) (Ballance and Pitman, 1996; Curry, 1997; Ballance and Pitman, 1998). As fisheries related mortalities (IWC, 1999; Collins *et al.*, 2002; Anderson, 2014), pollution (Preen, 1991; IWC, 1999; Freije, 2015) and habitat fragmentation (IWC, 1999; Baldwin *et al.*, 2004) continue to threaten regional populations; clarification of the taxonomic status of *Tursiops* sp. in this region has become a conservation concern.

In the present study we combine new *T. aduncus* mitogenomic sequences from the northwest Indian Ocean with the mitogenome dataset generated by Moura *et al.*, (2013). In addition, a dataset consisting of *T. aduncus* and *T. truncatus* samples from the northwest Indian Ocean and sequences from five mtDNA loci and two nuDNA loci were analysed to improve representation from the region and include bi-parentally inherited markers. We investigate whether ancestral distributions and divergence times at key phylogenetic nodes, particularly within the *T. aduncus* lineage, coincide with historic climatic events throughout the Pleistocene. In particular, we test the hypothesis that historical climate transitions during the Pleistocene are consistent with the timing and pattern of differentiation. Understanding this will provide important insight into the processes underlying the evolution of diversity in mobile marine taxa.

2. Material and methods

2.1. Sample Acquisition and DNA Extraction

Among the 98 samples included in phylogenetic reconstructions, representing various regional populations and putative species, new regions were represented by Oman, collected from strandings ($n = 1$) or free-ranging ($n = 7$) individuals and from strandings in Pakistan ($n = 2$; see Table S1). Samples from India ($n = 11$) were provided by the Environmental Specimen Bank (es-BANK) of Ehime University, Japan. All mitogenome sequences generated by Moura *et al.*, (2013) and two generated by Xiong *et al.*, (2009) were incorporated into the study (see Table S1 for locations and Table S2 for Accession Numbers). Figures 1 a) and b) show the geographic locations of samples. DNA extraction was carried out on all tissue samples using phenol-chloroform DNA extraction protocols, as adapted from Hoelzel (1998a).

2.2. Mitogenome Sequencing and Assembly

Mitogenome sequences were generated from one Oman and two Pakistan samples following the protocols in Moura *et al.*, (2013). DNA extractions were quantified using a Qubit Fluorometer (Life Technologies Inc.). Aliquots were made to a concentration of 10 ng/ μ l and randomly sheared to a range of 100-600 base pairs (bp) using a sonicator (Diagenode Biopruptor Pico). Fragment size distributions were checked on a Bioanalyzer (Agilent Technologies) and samples were concentrated to 20 μ l using a centrifugal evaporator. Dual indexed sequencing libraries were then prepared following protocols adapted from Meyer and Kircher (2010). Capture-enrichment of mitogenomic DNA was then performed on the libraries (500 ng) using a target-enrichment kit (MYbaits, MYcroarray Inc.). Bait probes were synthesised (20,000 probes, 100bp each, 2x coverage) with bait design based on an alignment of

killer whale, *Orcinus orca*, mitogenomes (Accession Numbers GU187171, GU187200, GU187194, GU187181, GU187209). Captured libraries were quantified using qPCR and pooled in equimolar concentrations. The final sample pool was quantified using the KAPA Universal qPCR quantification kit (KAPA Biosystems), validated on a TapeStation 2200 (Agilent Technologies) and then sequenced on the Illumina HiSeq 2500 in rapid run mode using 150 bp paired-end reads.

After sequencing, adapters were trimmed using the Reaper tool in Kraken v. 13-274 (Davis *et al.*, 2013) and de-multiplexing was carried out using the *process_radtags* program in Stacks v. 1.44 (Catchen *et al.*, 2013). Reads for each individual were then transferred to Geneious v. 7.1.2 (<http://www.geneious.com>, Kearse *et al.*, 2012) for quality trimming and assembly. Reads were mapped to a *T. aduncus* mitogenome reference sequence (GenBank Accession Number EU557092) using the algorithm available in Geneious. The Geneious map reader algorithm is a multi-step procedure which processes reads one at a time to match short sequences of 10 - 15 bp, 'words', to a reference sequence. Matched locations in the reference sequence are then used to 'seed' a mapping process that expands across the length of the read (see the user manual for details). Mapping was set to 'medium-low sensitivity/fast' with up to five iterations. Consensus sequences were generated using the '50% - Strict' threshold. A minimum depth of coverage threshold of 5X was used.

2.3. Amplification of mtDNA loci

To construct additional phylogenies based on both mtDNA and nuclear loci, an informative region of the mitochondrial genome comprising a total of 4,301 bp was sequenced for 21 individuals from Oman ($n=8$), Pakistan ($n=2$) and India ($n=11$). PCR amplifications were performed for five mtDNA fragments spanning five loci: the

control region, cytochrome-*b*, 12SrRNA, 16SrRNA and ND6. Primers ($n=9$; see Table S3) were designed in Primer3 v. 2.3.4 (Untergrasser, 2012) as implemented in Geneious, which were combined with previously published primers (see Table S3) for the final PCR amplification. All amplifications were performed in a 20 μ l final reaction volume containing 1.0 μ l of template DNA, 1.25 U of GoTaq Flexi DNA polymerase (Promega), 1x GoTaq Flexi buffer (Promega), 0.2 mM dNTP, 1-2 mM $MgCl_2$ and 0.16-0.2 μ M of each primer. The PCR temperature profile for each fragment included an initial heating step at 95°C for 2 min, followed by 45 cycles of 95°C for 30 s, annealing temperature for 40 s and 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were purified with QIAgen PCR purification columns (Qiagen, GmbH, Germany) and Sanger sequenced using an ABI automated sequencer. Primer sequences, annealing temperatures and product sizes are summarised in Table S3.

2.4. Amplification of *nuDNA* loci

Two nuclear loci were amplified, chosen based on good resolution in published multi-species phylogenies (Banguera-Hinestroza *et al.*, 2008; Caballero *et al.*, 2008; Banguera-Hinestroza *et al.*, 2014). A segment of 995 bp from Actin intron 1 and 472 bp from α -Lactalbumin intron 2 were amplified for 40 individuals (see Table S2). The Actin gene codes for a muscle protein whereas the α -Lactalbumin gene codes for a mammary secretory protein (Milinkovitch *et al.*, 1998; Harlin-Cognato and Honeycutt, 2006). A final reaction volume of 20 μ l contained 1.0 μ l of template DNA, 1.25 U of GoTaq Flexi DNA polymerase (Promega), 1x GoTaq Flexi buffer (Promega), 0.2 mM dNTP, 2 mM $MgCl_2$ and 0.16 μ M of each primer. The PCR temperature profile began with an initial denaturation step at 94°C for 2 min, followed

by 45 cycles at 92°C for 30 s, and annealing temperature for 30 s and an extension at 72°C for 30 s. A final extension step of 72°C for 5 min was also included. PCR products were purified and sequenced as above. See Table S3 for details.

2.5. Phylogeny Reconstruction

Two datasets were analysed: 1) mitogenomes and 2) concatenated mtDNA and nuDNA loci. For the mitogenome trees, extending the phylogeny published earlier (Moura *et al.*, 2013), our novel mitogenomes were aligned with database mitogenomes (see Table S2) using the MUSCLE algorithm (Edgar, 2004) as implemented in Geneious. For the combined mtDNA and nuclear locus phylogenies, each nuDNA locus was phased using the PHASE algorithm (Stephens and Donnelly, 2003; Stephens *et al.*, 2001) as implemented in DnaSP v5 (Librado and Rozas, 2009). As it was reasonable to assume no linkage between these different nuclear loci, they were concatenated randomly for each individual. Sequences of mtDNA for each individual were assigned to their respective nuDNA haplotypes and concatenated together. Where mitogenome sequences were available from Moura *et al.*, (2013), homologous mtDNA regions were excised and included in the combined nuDNA (1,467 bp) and mtDNA (4,301bp) phylogeny (see below and Table S2). Dusky dolphin (*Lagenorhynchus obscurus*) sequences, available from GenBank, were used as an outgroup (Table S2). All sequences were aligned using the MUSCLE algorithm (Edgar, 2004) as implemented in Geneious.

MrBayes v. 3.2.2 (Huelsenbeck and Ronquist, 2001) was implemented online using the CIPRES Scientific Gateway v. 3.3 (Miller *et al.*, 2010) to estimate separate phylogenies for the mitogenome dataset and the concatenated mtDNA/nuDNA dataset. Following Moura *et al.*, (2013), four independent MCMC were run for

22,000,000 iterations with a burn-in period of 2,200,000 iterations and a sampling frequency of 4,000 iterations. Three of the four chains were heated and the analysis was run twice. Convergence was confirmed through examination of various diagnostic outputs, particularly the ESS (Effective Sample Size) and PSRF (Potential Scale Reduction Factor) values. All ESS values were greater than 100 (minimum values ranged from 1741.93 - 4501.00) and all PSRF values approached one, indicative of convergence and that a sufficient number of generations had been implemented. The best partitioning scheme was inferred using the 'greedy' algorithm as implemented in PartitionFinder v. 1.0.1 (Lanfear *et al.*, 2012; 2014) considering the evolutionary models available to MrBayes. Substitution model and partitioning selection was carried out using the Bayesian Information Criterion (BIC) metric. Partitioning schemes are shown in Table S4.

A maximum likelihood (ML) phylogenetic tree was generated for both the mitogenome and concatenated mtDNA/nuDNA datasets using RaxML v. 8.0.24 (Stamatakis 2014) as implemented on CIPRES. The alignments were partitioned following the best partitioning scheme identified in PartitionFinder considering the evolutionary models available to RaxML. The best supported model was GTR (general-time-reversible) with gamma substitution rate heterogeneity (see Table S4) and this was applied across all partitions with individual alpha-shape parameters, GTR-rates, and empirical base frequencies optimized for each partition during analysis. Bootstrap node support values were generated over 5,000 iterations.

2.6. Congruence Between mtDNA and nuDNA Markers

To examine congruence between the mtDNA and nuDNA markers, partitioned Bremer support indices (PBSIs) (Baker and DeSalle, 1997) were calculated for each

node in a phylogeny generated from the concatenated mtDNA/nuDNA dataset in PAUP* v. 4.0b10 (Swofford, 2011). PBSIs are a measure of each locus' contribution to the estimated topology, whereby positive values indicate support for a node and negative values indicate the contrary in a combined analysis (Baker *et al.*, 1998). The sum of all PBSIs at a node is equal to the total Bremer support value for that node (Baker *et al.*, 1998). A heuristic maximum parsimony analysis was performed with Tree-Bisection-Reconnection branch swapping and 1,000 random-addition-sequence replications. The maximum number of saved trees 'maxtrees' was set to automatically increase by 100. Node support was obtained from 500 bootstrap replicates. Outgroups were defined as dusky dolphin and harbour porpoise using sequences available from GenBank (Table S2). All characters were unordered and equally weighted and a strict consensus phylogeny was generated from the tree output. This phylogeny was used to generate a PAUP* command file which was subsequently run in TreeRot v. 3 (Sorenson and Franzosa, 2007). PBSI values were parsed from the output in TreeRot and plotted on a 50% majority-rule consensus phylogeny based on the heuristic analysis.

2.7. Reconstruction of Ancestral Distributions

To reconstruct the biogeographic state of ancestral nodes, statistical dispersal-vicariance analysis (S-DIVA; Ronquist, 1997) was implemented in RASP v.2.2 (Yu *et al.*, 2010). We randomly sampled 10,000 trees from a Bayesian phylogenetic Markov Chain Monte Carlo (MCMC) analysis, generated from a mitogenome alignment including all *Tursiops* individuals and a rough-toothed dolphin (*Steno bredanensis*; Accession Number JF339982) as outgroup. S-DIVA analysis was run on all trees, and results were plotted on a majority-rule consensus tree, generated from

the MCMC output in RASP. Sampling locations were used to provide populations with unique distributions (see Fig. 1). Following Moura *et al.*, (2013), a further distinction was made between coastal vs pelagic ecotypes. Both the Australasian *T. aduncus* (including individuals from China) and the Burrnun dolphin *T. australis* were considered as occupying Australasia. The maximum number of areas considered for each node was constrained to four in order to limit the number of possible distribution regions assigned to ancestral nodes. This is because optimization of ancestral areas becomes less reliable as we approach the root node (Ronquist, 1996). The outgroup was assigned a null distribution by using a location unique to it.

A Bayesian Binary MCMC (BBM) analysis was also performed in RASP using the same dataset. A null root distribution was assigned to the outgroup and a maximum of four areas for each node was configured. The BBM analysis was run for 5,000,000 iterations with a burn-in of 5,000. The sampling frequency was set to 100, and 10 chains were run with a temperature of 0.1. The Fixed Jukes-Cantor model for state frequencies was applied with the gamma shape parameter for among-site rate variation. The analysis was run twice to check for convergence. Both S-DIVA and BBM analyses were repeated on a Bayesian phylogeny derived from the concatenated mtDNA and nuDNA dataset.

2.8. Estimates of Divergence Dates using Mitogenomic Data

Divergence dates were estimated from the mitogenome dataset using a partitioned analysis using BEAST v.1.8 (Drummond and Rambaut, 2007). Eight partitions were identified in the data (see Table S4 for details and evolutionary models). Using a very similar dataset, Moura *et al.*, (2013) performed rigorous model testing using different tree priors (including coalescent priors), different clocks and

different calibration points (and associated priors). The model that performed optimally, based on Bayes factors, considered a Yule Process tree prior, an exponential relaxed clock and only a biogeographic calibration point, defined according to the opening of the Bosphorous Strait. However, this model resulted in divergence dates that were inconsistent with older divergence times reported in other studies and the fossil record (Moura *et al.*, 2013). Similarly, models considering only fossil calibrations resulted in divergence times that were too old. The model that Moura *et al.*, (2013) considered the best was a ‘total evidence’ model, which performed well (based on Bayes factors) but also provided inference that was consistent with geological data and published mutation rates.

Therefore, in light of the more extensive model testing performed in Moura *et al.*, (2013) we only consider three of their models (see Table S5) to determine whether our inference was different to that reported in Moura *et al.*, (2013). We consider: 1) the best, ‘total evidence’, model, 2) the most optimal model (which only considers biogeographic calibration nodes) and 3) a model which considers only fossil calibration nodes.

For all models, the initial tree was generated at random, the exponential distribution of mutations model was used for the uncorrelated relaxed clock model and the tree prior followed a Yule branching model (following Moura *et al.*, 2013). For models 1 and 2, the two terminal clades including Eastern Mediterranean and Black Sea groups (BSEM) were each constrained to monophyly with the same time to most recent common ancestor (TMRCA) priors. The TMRCA priors for these nodes were given a uniform distribution between 3 and 10 Ka, consistent with the opening of the Bosphorous Strait (see Moura *et al.*, 2013). For models 1 and 3, two fossil calibration points were also used; the TMRCA for Delphinoidea (McGowen *et al.*,

2009; Steeman *et al.*, 2009; Xiong *et al.*, 2009) and the TMRCA for the clade that includes all *Tursiops* species (Barnes, 1990; Fitzgerald, 2005). The ancestor to Delphinoidea was defined by constraining the clade that includes Monodontidae and Delphinidae to monophyly, and the *Tursiops* ancestor was defined by constraining the clade that included all *Tursiops*, and other delphinids nested within that group, to monophyly. Normal distributions were assigned to both fossil TMRCA priors, with means of 10 Ma for the Delphinoidea ancestor and 5 Ma for the *Tursiops* ancestor, each with a standard deviation of 1.5 Ma (see Table S5).

For all models, MCMC analyses were run with 150 million iterations with 10% burn-in, sampling every 5,000 generations. Convergence was confirmed by examining the posterior probability distributions of parameters from the different runs in TRACER v.1.6 (Rambaut *et al.*, 2014). ESS values for most parameters exceeded 200 for individual runs, suggesting an appropriate number of iterations had been performed. All ESS values exceeded 200 when individual runs were combined in LogCombiner v. 1.7.5 (Drummond and Rambaut, 2007). Trees from the different runs were similarly combined and resampled at a lower frequency of 60,000 runs, yielding 9,000 trees, for each model. These trees were summarised in TreeAnnotator v.1.7.5 (Drummond and Rambaut, 2007).

Models were compared using stepping-stone sampling (Xie *et al.*, 2011), which is the most reliable means available of estimating marginal likelihoods for model comparison (Baele *et al.*, 2013). For each model, four independent runs were performed with 100 power-posteriors run for 1,000,000 iterations. Stepping-stone sampling was then used to estimate the log marginal likelihoods from the combined outputs (Baele *et al.*, 2012; 2013). Log Bayes factors were generated from the log

marginal likelihoods for model comparison. To check log marginal likelihoods were converging, the runs were carried out again for longer (2,000,000 iterations).

3. Results

3.1. Phylogenetic Reconstructions

The Bayesian (Fig. 2 - 3) and ML (Fig. S1 - S2) phylogenies showed similar topologies for each dataset, and phylogenies generated from the different datasets (concatenated mtDNA/nuDNA vs mitogenomes) also had similar topologies. The maximum parsimony tree for the mtDNA/nuDNA dataset also has similar topology (see Fig. 4). In the combined mtDNA and nDNA phylogenies, mtDNA provided the stronger inference (Fig.s 4, S4, S5). Comparing lineages, divergence of the new *T. aduncus* lineage from the Australasian lineage (node 'd' in Fig. 4) is 1.79% and from the holotype lineage is 2.12% (node 'c'). The holotype lineage and Australasian lineage diverge by 2.04% (node 'b').

The values for key nodes from the concatenated mtDNA/nuDNA phylogeny PBSIs are presented in Fig. 4. The majority of loci were consistent in their node support, and where not, PBSI values were > -0.2 (Fig. 4). There were four and ten segregating sites for Actin and α -Lactalbumin, respectively, and PBSIs ranged between -0.14 and 2.9 for these loci. The mtDNA loci generally showed stronger support for nodes (up to a PBSI value of 22.8 for the cytochrome-*b* locus at node 'a'; see Fig. S4), though there was some positive support from nuclear loci as well across the tree (Fig. 4, S5). Both nuDNA loci supported the nodes between *T. australis* and the broader *Tursiops* lineage (node 'a'), between the *T. aduncus* and *T. truncatus* lineages (node 'b'), and between the *T. aduncus* holotype lineage and the broader *T. aduncus* lineage (node c). Where PBSI values were low, there were only slight

deviations from a PBSI = 0 (min = -0.14), indicating that all loci were either congruent or uninformative in their support for key divergence events across the *Tursiops* lineage.

3.2. Reconstruction of Ancestral Distributions

For the mitogenome tree the biogeographic distribution of the ancestor to *T. aduncus* and *T. truncatus* (Node 157, Fig. 5a) is unresolved based on the S-DIVA analysis, however, the BBM analysis suggests Australasia as most likely (55.97%) (Node 157, Fig. 5b). The origin of the *T. aduncus* lineage (Node 109) is also unresolved in the S-DIVA analysis (Fig. 5a), however again the BBM analysis (Fig. 5b) suggests Australasia as most likely (47.47%). The S-DIVA analysis strongly indicates that the ancestral origin of the Australasian and Arabian Sea lineages (Node 108) is Australasia/Pakistan (100% support) (Fig. 5a) while the BBM analysis indicates an Australasian origin (77.59% support) (Fig. 5b). Although the BBM analysis does not distinguish between dispersal and vicariance for any of the key nodes, the S-DIVA analysis indicates that both the node separating the Australasian and holotype lineages (node 109) and the node separating the Australasian and Arabian Sea lineages (node 108) were likely vicariant events.

In reconstructions generated from the concatenated mtDNA-nuDNA sequences, the S-DIVA (Fig. S3a) and BBM (Fig. S3b) results are largely congruent with those derived from the mitogenome dataset (Fig. 5). An Australasian origin for the ancestor to all extant *Tursiops* species and ecotypes is supported. Furthermore, an Australasian distribution is supported for the ancestors common to all extant *T. aduncus* (Nodes 108 and 109) and the ancestor to *T. aduncus* and *T. truncatus* (Node 157). BBM reconstructions using the concatenated mtDNA-nuDNA phylogeny

support the hypothesis that *T. truncatus* ancestors were a coastal ecotype. Nodes 108 and 109 are again supported as vicariance events by S-DIVA in this tree.

3.3. Estimates of Divergence Dates using Mitogenomic Data

Inferred node dates for the ‘total evidence’ model (model 1) were congruent with those estimated in Moura *et al.*, (2013) (see Fig. 6 and Table S6). Within *T. aduncus*, the holotype lineage diverged from other *T. aduncus* ~342 Ka (95% HPD: 143, 630 Ka) and divergence of the Australasian and Arabian Sea lineages was estimated to have occurred ~ 261 Ka (95% HPD: 111, 509). Comparison of the three models using log Bayes factors suggested that model 1, which was the Moura *et al.* (2013) ‘total evidence’ model that included both fossil and biogeographic calibrations, outperformed the others (see Table S7). Our use of stepping-stone sampling to estimate log marginal likelihoods (distinct from Moura *et al.* 2013), has been suggested to be the most robust method (Baele *et al.*, 2013).

4. Discussion

During the Pleistocene, the effects of climate change on sea level and oceanographic properties were substantial across the Indo-Pacific (Kassler, 1973; Fontugne and Duplessy, 1986; Shackleton, 1987; Wang *et al.*, 1999a; Almogi-Labin *et al.*, 2000; Voris, 2000; Sun *et al.*, 2003; Bailey, 2009; Gaither and Rocha, 2013). The contemporary oceanography in the Indian Ocean is also particularly heterogeneous, harbouring potential environmental breaks (discontinuities) (e.g. Mendez *et al.*, 2011) and therefore opportunities for resource polymorphisms to develop (Skúlason and Smith, 1995; Hoelzel, 1998b). These factors are likely to contribute to population and taxonomic structure across various marine taxa in the

region, e.g. reef fish (Bay *et al.*, 2004; Gaither *et al.*, 2011; Hubert *et al.*, 2012), gastropods (Crandall *et al.*, 2008), sea stars (Williams and Benzie, 1998) and cetaceans (Jefferson and Van Waerebeek, 2002, 2004; Mendez *et al.*, 2011, 2013; Pomilla *et al.*, 2014).

Using samples obtained from the northwest Indian Ocean, we provide evidence for a new lineage of *T. aduncus* that is closely related to the Australasian *T. aduncus* lineage. The mtDNA/nuDNA phylogeny, where sample representation from the region is greatest, shows that the new lineage (hereafter referred to as the Arabian Sea lineage) can be found off Oman, Pakistan and India (Fig. 3 and Fig. S2). We also confirm the presence of *T. truncatus* among samples collected in India and Oman, and show that they group with the broader pelagic and European coastal populations, suggesting incomplete lineage sorting. Reconstruction of ancestral biogeography revealed Australasia as the most likely origin for several *Tursiops* lineages within the lower Pleistocene (as reported previously by Moura *et al.*, 2013). Here we show that the holotype *T. aduncus* lineage diverged from other *T. aduncus* ~342 Ka (95% HPD: 143, 630 Ka) and the Australasian and Arabian Sea lineages diverged ~261 Ka (95% HPD: 111, 509). While we cannot confirm whether these occurred during glacials or interglacials, due to large credible intervals, the relative ~100 Ka periodicity of divergence events is consistent with glacial oscillations (Gildor and Tziperman, 2000; Rohling *et al.*, 2014). From this, it seems apparent that events in Australasia during the Pleistocene were important in driving multiple divergence events in *Tursiops*, and possibly other closely related delphinids in the region (e.g. Mendez *et al.*, 2013).

The range of the newly described Arabian Sea lineage evidently overlaps with that of the holotype lineage, as both are found in Oman and India, which is suggestive of secondary contact or sympatric/parapatric divergence in the northwest Indian Ocean.

In order to explain the presence of three distinct *T. aduncus* lineages in the Indo-Pacific, we need to consider two systems: one driving multiple allopatric divergence events in Australasia followed by recolonisations, and the other facilitating sympatric divergence and maintenance of reproductive isolation in the northwest Indian Ocean. During glacial periods, exposure of the Sunda and Sahul shelves (Voris, 2000) in Australasia caused the contraction of suitable habitat between the eastern Indian Ocean and the western Pacific (Gaither and Rocha, 2013), establishing the conditions for allopatric divergence, impeding gene flow between once adjacent populations. Various studies have implicated this barrier as a factor promoting marine species diversity in that part of the world (e.g. Bay *et al.*, 2004; Gaither *et al.*, 2011; Hubert *et al.*, 2012; Gaither and Rocha, 2013).

The nature of a putative barrier and a divergence process in the northwest Indian Ocean is less clear. There is some evidence to suggest that the Sea of Oman coastline could provide a barrier off Oman (see Baldwin *et al.*, 2004). However, individuals that group with the holotype lineage have been found either side of this barrier, in the Arabian Gulf and Arabian Sea (Gray, 2016), so present day habitat differences between the Arabian Sea and Sea of Oman coasts are, at least, not a strict barrier off Oman. The distributional overlap between the holotype and Arabian Sea lineages could be construed as secondary contact between lineages that diverged in allopatry following the recent disappearance of a historic barrier. Palaeoclimatic and palaeoproductivity data suggest there was great variability in the monsoon systems during the Pleistocene. In contrast to today, the northeast and East Asian monsoons intensified and were the dominant feature during certain glacial events, while the southwest monsoons weakened (Fontugne and Duplessy, 1986; Wang *et al.*, 1999a; Almogi-Labin *et al.*, 2000; Sun *et al.*, 2003). These changes may have altered the

distributions of available prey and habitat, creating an ecological barrier in the northwest Indian Ocean.

Alternatively, divergence may have occurred in sympatry driven by environmental heterogeneity and associated discontinuities in the region, perhaps resulting in local adaptation through the acquisition of resource polymorphisms (such as foraging specialisations; Skúlason and Smith 1995; Hoelzel 1998b). This process may also continue to reinforce lineages that diverged in allopatry and are currently in secondary contact (see above). Briggs and Bowen (2012) delineate marine biogeographic provinces based on fish endemism and show the region from the central Indian Ocean to the eastern limits of the Western Pacific to be a separate province from the western Indian Ocean. These differences in fish species assemblages may be indicative of different prey compositions available to the different *T. aduncus* lineages occupying them.

The processes discussed above (Fig. 7a) imply that the Arabian Sea lineage is more closely related to the holotype lineage than to the Australasian lineage, which is at least superficially incongruent with the phylogeny estimated here. However, during the interglacial that followed the first divergence event (~342 Ka) more introgression may have occurred between populations experiencing secondary contact across the Indo-Pacific boundary than across the putative barrier in the northwest Indian Ocean, resulting in the Arabian Sea lineage having a closer phylogenetic affinity to the Australasian lineage than to the holotype lineage. An alternative process, whereby populations in the east displaced those in the west during interglacial periods (see Fig. 7b), could also explain the phylogenetic pattern. However, to the extent that the exhibition of habitat preferences and site fidelity (as is the tendency for this species; e.g. Gross *et al.*, 2009; Moura *et al.*, 2013) was also an ancestral trait, this mechanism

seems less credible. Given the recent divergence in these lineages, it is important to also note that the tree topology may not reflect the true relationships due to incomplete lineage sorting (especially for inference dominated by mtDNA data).

It is interesting to note that *Sousa* spp., a closely related delphinid that shares coastal habitat with *T. aduncus* (Wang and Yang, 2009), shows a similar phylogeographic pattern, with three putative lineages across the Indian Ocean (Mendez *et al.*, 2013). Jefferson and Van Waerebeek (2002) propose a similar process for the divergence of the common dolphin *D. capensis tropicalis*, which also occurs in waters off the northwest and northern Indian Ocean.

Being a coastal cetacean, *T. aduncus* is under particular threat in the northwest Indian Ocean from an expanding fisheries industry (Salm *et al.*, 1993; IWC, 1999; Collins *et al.*, 2002; Anderson, 2014), pollution (Preen, 1991; Freije, 2015), and habitat degradation (IWC, 1999; Baldwin *et al.*, 2004). Although there is national and international legislation in place across much of the region to prevent hunting/trade of dolphins (e.g. IWC, CITES), there are no management strategies currently in place to address indirect impacts on dolphin populations (Ponnampalam, 2009). The identification of a previously unrecognized, monophyletic lineage in the northern Indian Ocean (the Arabian Sea lineage) is an important step towards resolving bottlenose dolphin taxonomy in the region (IWC, 1999; Reeves *et al.*, 2004; IWC, 2016), and will have important conservation implications. Especially important is the fact that a minority of samples collected off Oman and off India fall into two different genetic lineages of *T. aduncus*, which implies some degree of range overlap across the Arabian Sea and Sea of Oman, and a need to manage mixed assemblages.

5. Conclusions

As outlined above, the Pleistocene altered the spatio-temporal distribution of available habitats, and the taxa that occupied them, such that populations could differentiate by vicariance (Hofreiter and Stewart, 2009; Stewart *et al.*, 2010). In the marine environment, variation in the Asian monsoon systems during the Pleistocene may have driven phylogenetic structure in regional marine taxa, such as the spiny lobster, *Panulirus homarus* (Pollock, 1993). Exposure of a land bridge in Australasia, during low sea level stands, formed a physical barrier between the Indian and Pacific Oceans. This barrier has been implicated in the phylogeographic patterns observed in several reef fish species (Gaither and Rocha, 2013), such as the peacock grouper, *Cephalopholis argus* (Gaither *et al.*, 2011). In the northern Indian Ocean, higher turbidite deposits from the Indus delta during glacial periods suggest the environment may have been particularly turbid (von Rad and Tahir, 1997). River deltas in the region, such as the Ganges and Indus, may prove credible candidates for barriers to dispersal. For example, the Amazon delta has been implicated in the phylogeographic pattern exhibited in several Atlantic reef fish (Rocha *et al.*, 2002; Floeter *et al.*, 2008). Here we show that the distribution and timing of differentiation within the genus *Tursiops*, particularly within the *T. aduncus* lineage which relies on coastal habitat, could be consistent with these same processes, and reveal a newly discovered evolutionary significant unit within this radiation.

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Figure Captions

Fig1_Map_FINAL.pdf [Figure]

Fig. 1 a) Sample locations from worldwide populations of bottlenose dolphins (*Tursiops* spp.). Tt = *Tursiops truncatus*; Ta = *Tursiops aduncus*; GC = Gulf of California; WNAC = northwest Atlantic (coastal ecotype); WNAP = northwest Atlantic (pelagic ecotype); SCO = Scotland; EMED = eastern Mediterranean; BSEA = Black Sea; OM = Oman; PAK = Pakistan; IND = India; SA = South Africa; SABD = Burrunan dolphin, *T. australis*; AUS = Australasian Indo-Pacific bottlenose dolphin; CHINA = Australasia (China); Rectangle delineates study area. b) Approximate locations of novel samples analysed in our study. Filled circles = known sample locations; Open circles = unknown sample locations from respective country; numbers = sample numbers associated with each circle. Indian samples were collected within the following grid cell: 7°15' 59.2" N - 62° 38' 53.61" E to 32° 59' 13.54" N - 88° 17' 31.53" E.

Fig2_Bayesian_Phylogeny_Mtgenome.pdf [Figure]

Fig. 2 Bayesian phylogeny inferred from the mitogenome dataset using MrBayes v. 3.2.2 (Huelsenbeck & Ronquist 2001). Note that posterior probabilities less than 1 are shown at respective nodes, and that nodes without a value shown all have the value of 1. Scale bar=substitutions/site.

Fig3_Bayesian_Phylogeny_mtDNA_nuDNA.pdf [Figure]

Fig. 3 Bayesian inferred phylogeny generated from concatenated mtDNA/nuDNA dataset. Note that posterior probabilities less than 1 are shown at respective nodes, and that nodes without a value shown all have the value of 1. Proportional transformation applied to the branch lengths to emphasise tree topology.

Fig4_Max_Parsimony_TreeRot.pdf [Figure]

Fig. 4 Maximum parsimony tree and partitioned Bremer support indices for different loci. Mitochondrial markers: ND6, Cytochrome-b, D-loop, 12SrRNA and 16SrRNA. Nuclear DNA markers: Acting intron 1 and α -Lactalbumin intron 2. Nodes and charts: a) divergence of *T. australis* from other *Tursiops* species; b) divergence of *T. truncatus* and *T. aduncus* lineages; c) divergence of *T. aduncus* holotype lineage from other *T. aduncus* lineages; d) divergence of Australasian and novel *T. aduncus* lineages. Bootstrap support values less than 100 are indicated at respective nodes, and all other nodes have a value of 100.

Fig5_Mitogenomes_S-DIVA_BBM.pdf [Figure]

Fig 5. a) Statistical Dispersal Vicariance Analysis (S-DIVA) and b) Bayesian Binary MCMC Analysis (BBM) for mitogenome dataset as implemented in RASP v.2.2 (Yu *et al.* 2010). Nodes of interest are indicated by a small black circle with a unique number. Colours and letters correspond to the locations of the various populations, ecotypes and species represented in the tree. Black bars = divergence by dispersal; white bars=divergence by vicariance; grey bars = divergence by both dispersal and vicariance. Under each analysis, each node of interest has its own table showing the likely biogeographic reconstructions.

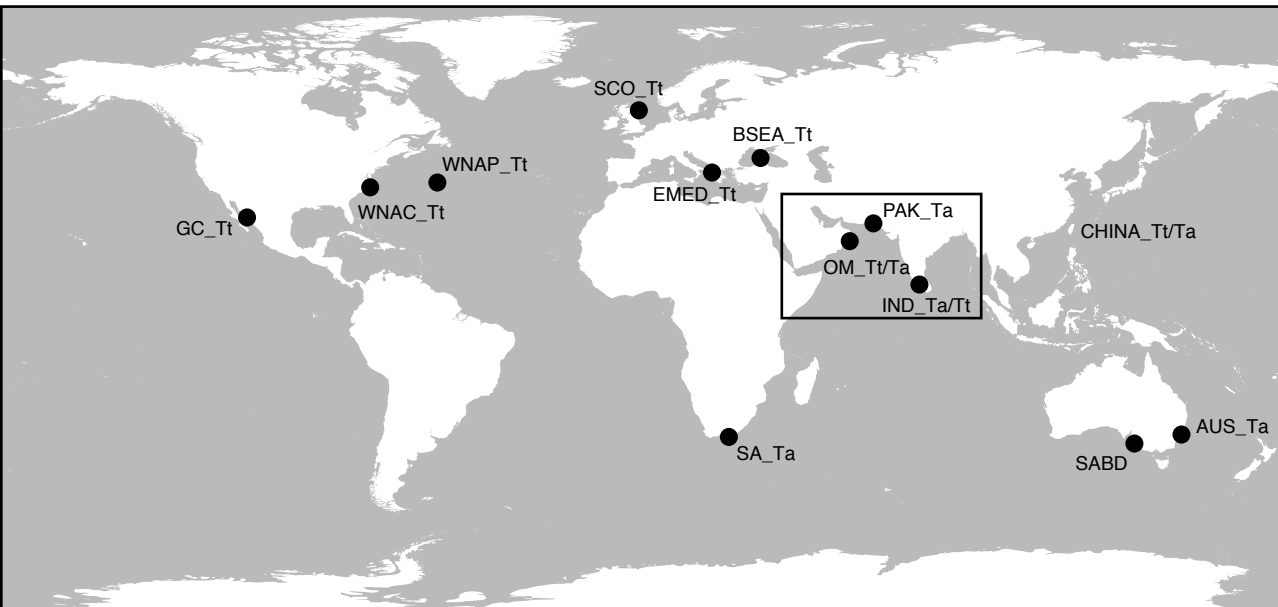
Fig6_BEAST_Tree.pdf [Figure]

Fig. 6 Estimation of divergence dates in BEAST v.1.8 (Drummond & Rambaut 2007). Divergence times indicated next to respective nodes. Grey bar indicates 95% highest posterior densities. Branch lengths are in Ka units according to the scale bar.

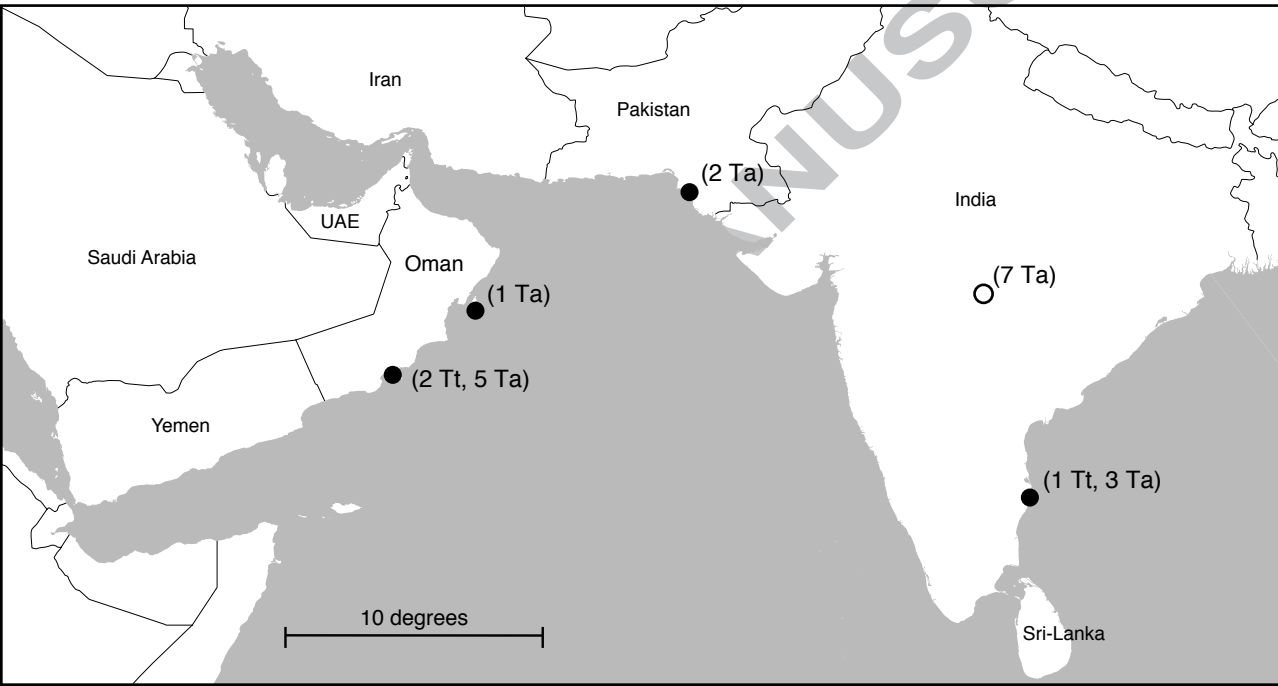
Fig7_Putative_Mechanism.pdf [Figure]

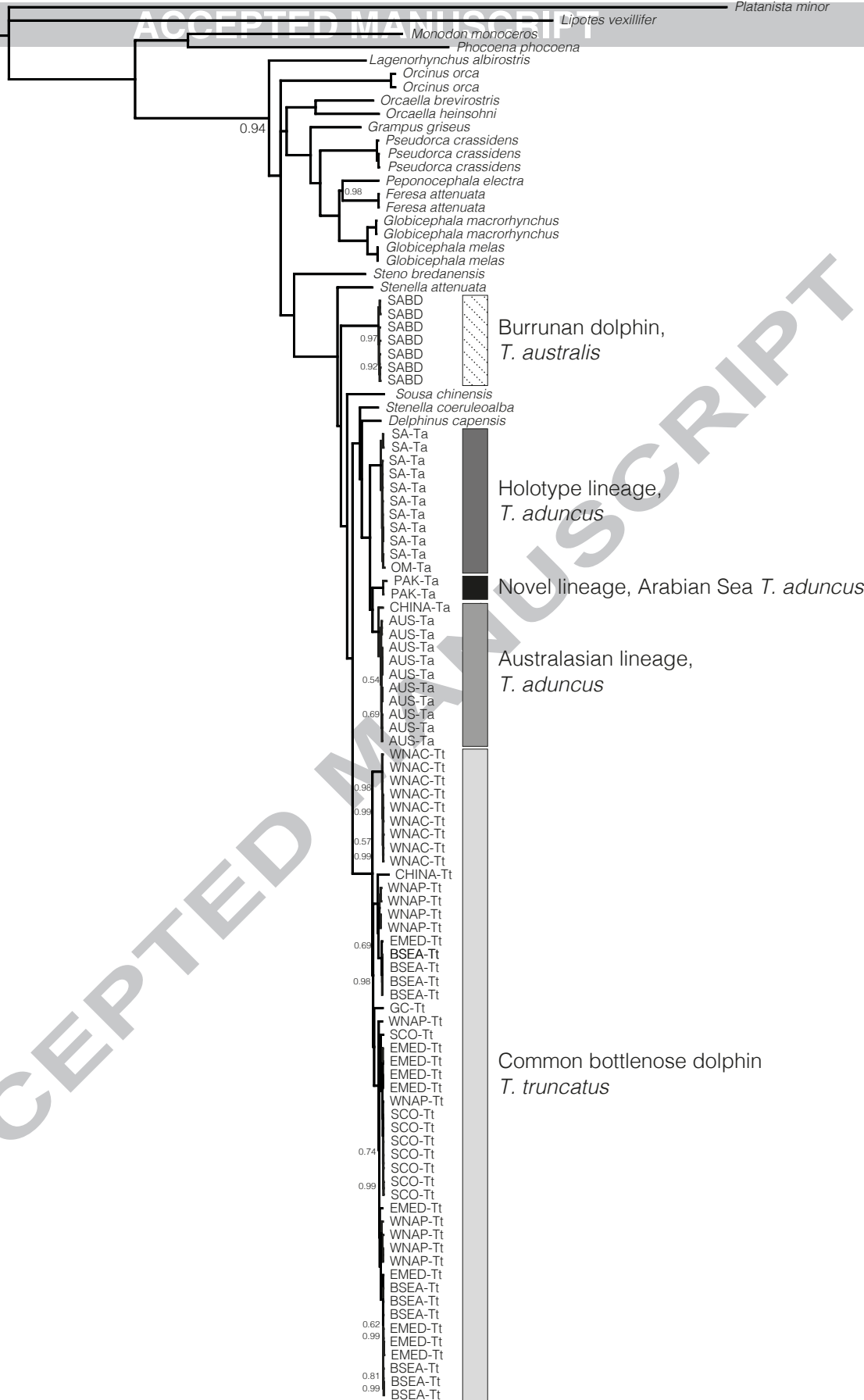
Fig. 7 Two proposed processes; a and b, for divergence events within *T. aduncus*. Black arrows indicate the direction of movement of dolphins. White arrow indicates the location of a putative physical or ecological barrier in the northwest Indian Ocean. The timing of movement across this barrier, illustrated during the interglacial in panels 3a and 3b, is unknown. Note the gradient in colour across the transitional zone between the Holotype and the Arabian Sea lineages to illustrate, approximately, where they occur in sympatry.

a)

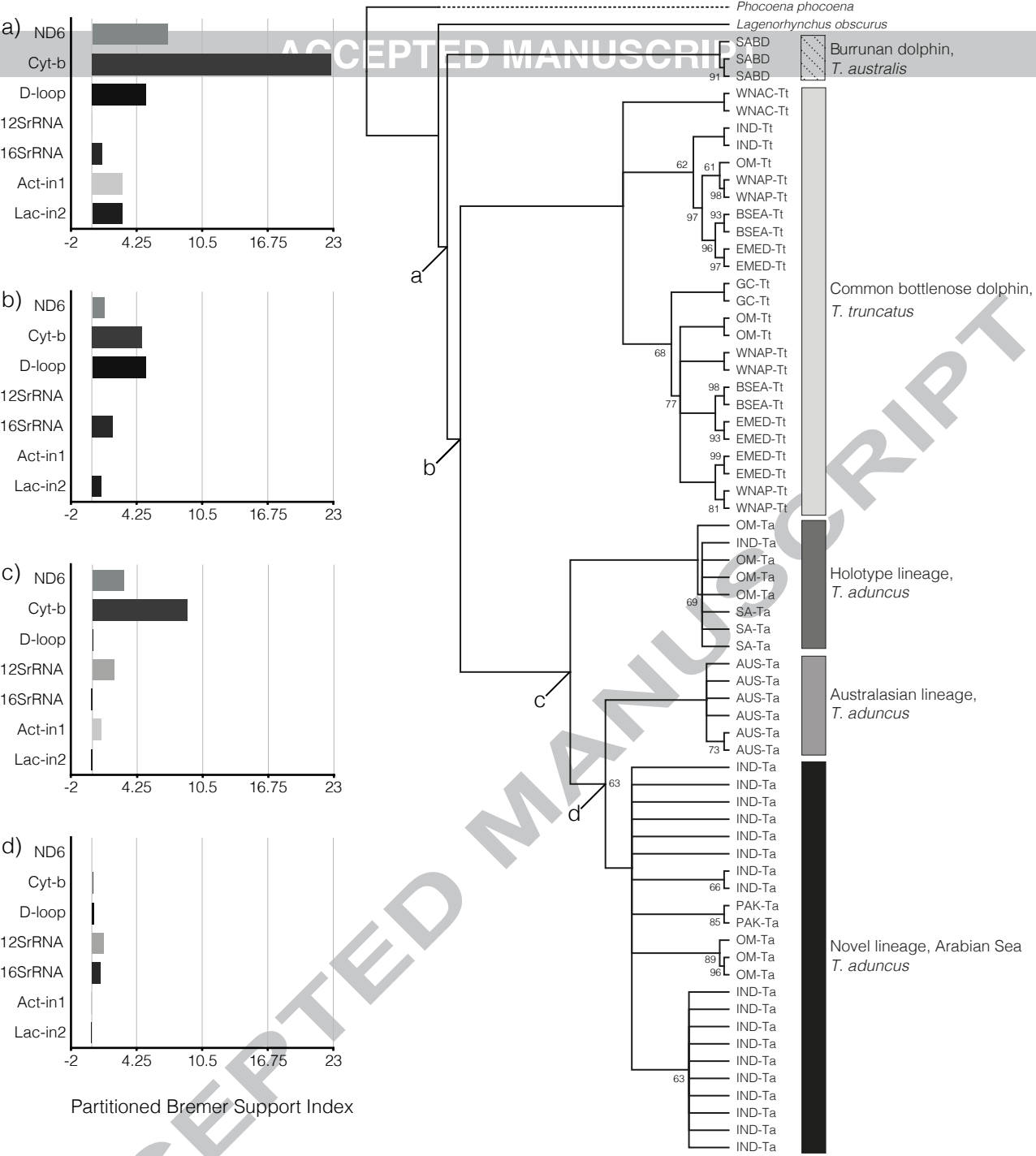


b)







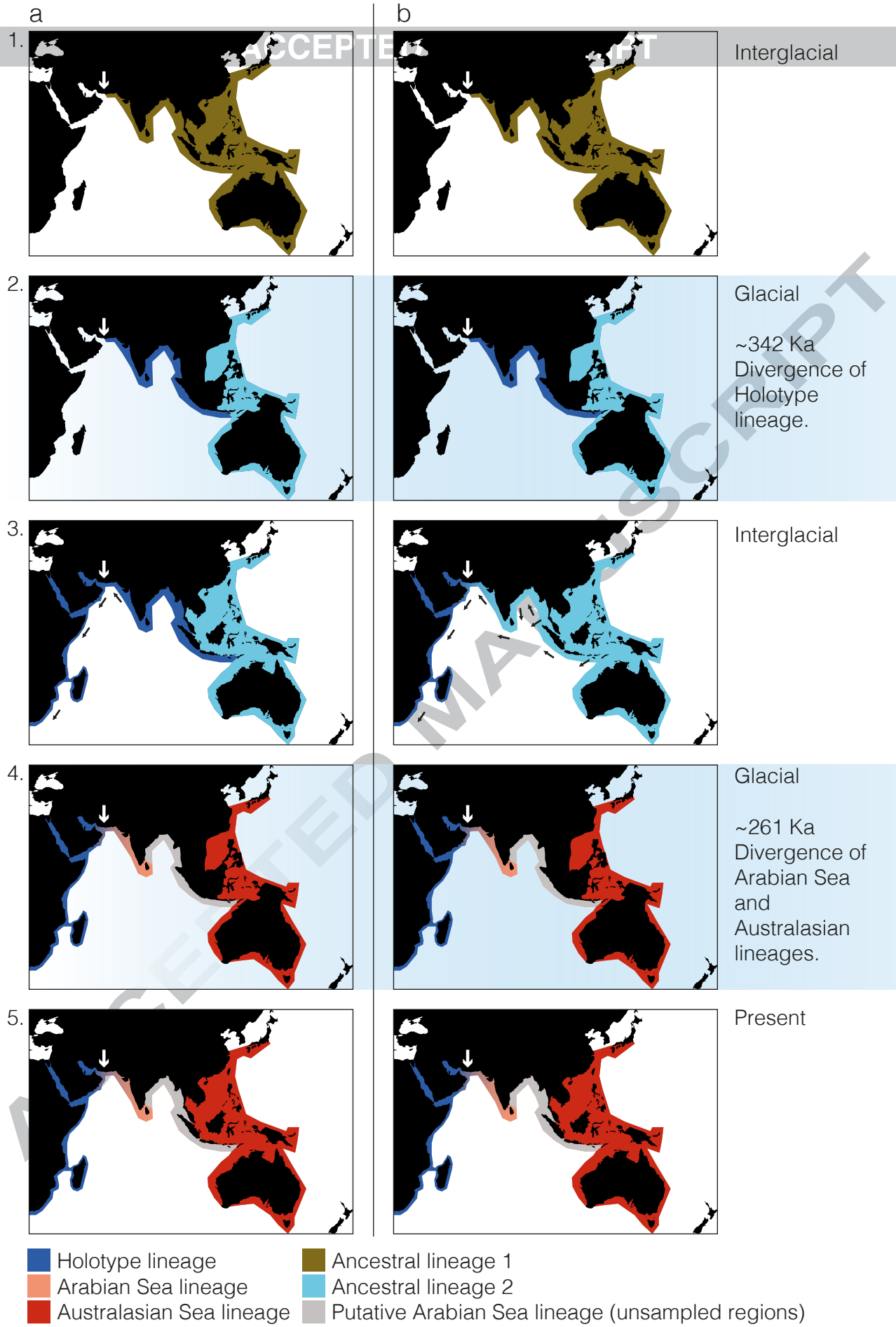


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Responsibility	Percentage
Current government	75%
Opposition	25%

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500



Highlights

- Discovery of a new lineage of *Tursiops aduncus* in the Indian Ocean.
- Divergence times within *Tursiops aduncus* coincide with Pleistocene glacial periods.
- Vicariance events in the northwest Indian Ocean and Australasia are proposed.

